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(54) Title: MOLECULAR DETECTION OF CHROMOSOME ABERRATIONS		
(57) Abstract The invention relates to the field of cytogenetics and the application of genetic diagnostic techniques in pathology and haematology. Specifically, the invention relates to nucleic acid probes that can be used in hybridisation techniques for the detection of chromosomal aberrations and other gene rearrangements such as immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements. The probes provided by the invention are a distinct and balanced pair of probes of comparable size each preferably being from 1 to 100 kb, or smaller, and flanking a potential breakpoint in a chromosome.		

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Title: Molecular detection of chromosome aberrations.

The invention relates to the field of cytogenetics and the application of genetic diagnostic techniques in pathology and haematology. Specifically, the invention relates to nucleic acid probes that can be used in
5 hybridisation techniques for the detection of chromosomal aberrations and other gene rearrangements such as immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements.

Chromosomal aberrations are a leading cause of
10 genetic disorders or diseases, including congenital disorders and acquired diseases such as malignancies. At the basis of the above malignancies lies the fact that all cells of a malignancy have a common clonal origin. Chromosomal aberrations in malignancies stem from rearrangements,
15 translocations, inversions, insertions, deletions and other mutations of chromosomes, but also losses or gains of whole chromosomes are found in malignancies. In many chromosome aberrations two different chromosomes are involved. In this way, genes, or fragments of genes are removed from the normal
20 physiological context of a particular chromosome and are located to a recipient chromosome, adjacent to non-related genes or fragments of genes (often oncogenes or proto-oncogenes). Such an aberrant genetic combination can be the foundation of a malignancy.

25 Often, such rearrangements involving two non-aberrant chromosomes happen in a somewhat established pattern. Breaks occur in either of the two chromosomes at a potential breakpoint or breakpoint cluster region resulting in removal of a gene or gene fragment from one chromosome and subsequent
30 translocation to the other chromosome thereby forming a rearranged chromosome where the rearranged fragments are fused in a fusion region.

Detection of chromosome aberrations can be achieved using a wide array of techniques, various of which entail modern biomolecular technology. Traditional techniques such as cytogenetic analyses by conventional chromosome banding techniques are, although highly precise, very labour intensive, require skilled personal and are expensive. Automated karyotyping is useful for some diagnostic applications, such as prenatal diagnosis, but is ineffective in analysing the complex chromosomal aberrations of many malignancies. Furthermore, above techniques require fresh (cultured) cells, which are not always available.

Other, more modern, techniques are Southern blotting or other nucleic acid hybridisation techniques or amplification techniques such as PCR, for the detection of well-defined chromosome aberrations for which suitable nucleic acid probes or primers are available. With these techniques, fresh or frozen cells can be used, and sometimes even samples after formalin fixation as long as the nucleic acid sequences to be hybridised or amplified remain intact and accessible. However, even with above modern technology, several disadvantages can be found that hamper the application of these diagnostic techniques in the rapid screening for chromosomal aberrations related to said malignancies.

Southern blotting lasts 3 to 4 weeks, which is too slow for efficient diagnosis and choice of therapy in malignancies, and allows only 10-15 kb of nucleic acid sequences to be analysed per probe analysis.

PCR, although in essence well-suited for rapid and massive diagnostic testing or even screening, allows only 0.1 to 2 kb of nucleic acid to be analysed per PCR analysis, which greatly hampers rapid screening of vast stretches of chromosomes and breakpoint cluster regions within the chromosomes. An additional disadvantage of PCR is its

inherent sensibility to mismatched primers. Small, normal, and physiological alterations which can always be present in the nucleic acid sequence of the gene fragment complementary to the primer hamper the reliable application of PCR and eventually give rise to false-negative results. Especially false-negative results render a PCR-based diagnostic test, albeit very specific, not sensitive enough for reliable diagnosis, and it goes without saying that only a reliable diagnosis of malignancies can contribute to an understanding of the prognosis and the design of an adequate therapy.

Fluorescent *in situ* hybridisation techniques (FISH) are less dependent on the complete matching of nucleic acid sequences to get positive diagnostic results. In general FISH employs probe analyses with large, mainly unspecified, nucleic acid probes that hybridise, however often with varying stringency, with the genes or gene fragments located at both sides of the fusion region in the rearranged chromosome in the malignant cell. Using large probes renders the FISH technique very sensitive. The binding of the colocalizing probes is generally detected either directly or indirectly with fluorochromes and visualised via fluorescence microscopy of a population of cells obtained from the sample to be tested.

However, even the currently used FISH protocols have inherent disadvantages, these mainly relate to the selection of nucleic acid probes employed in the current FISH protocols, which can give false-positive results in the diagnosis of chromosomal aberrations. For example, probes directed against different chromosomes with juxtaposition of signals in case of translocation create a rather large risk of false-positive results. Hence, the diagnostic tests are, albeit sensitive, not specific enough to employ standard FISH techniques in massive or rapid diagnostic testing, let alone in automated testing or screening.

Thus far, generally large probes, derived of cosmid clones, YAC clones, or other cloned DNA fragments have been used as probes in FISH. The exact position of these probes in relation to the fusion region in the rearranged chromosome is unknown and they are of largely unspecified and varying genomic length (genomic length or distance as expressed as the number of nucleotides or bases (b)) and go without specific selection or modification of these probes beyond the mere labeling of the probes with the necessary reporter molecules i.e. fluorochromes. For designing or selecting probes, little or none guidance is given in the art, beyond mere suggestions as to where to localise putative probe. False-positive results obtained with these probes may stem from aspecific hybridisation with a wide array of (major) repetitive sequences present throughout various chromosomes, or from cross-hybridisation to homologous sequences in the genome, or from overlap of the used probes with the breakpoint cluster region or from the difference in signal intensities as far as originating from size differences of the probes. These causes of false-positive results are frequently not recognized. False-positive results are especially detrimental to rapid diagnosis if rapid or routine screening of patients is needed to detect malignancies or in evaluating treatment protocols. A false-positive result then necessitates cumbersome re-testing of patients, or even unsuspecting clients that have been submitted to routine screening protocols, and can greatly alarm these people. Furthermore, translocations are generally detected with two different probes, one for each of the involved chromosomes, which probes than colocalise during the *in situ* hybridisation in case of a translocation, but show separate signals when no translocation is present (see for example EP 0430402, EP 0500290 A, Tkachuk et al., Science 250, 559-562, 1990; Tkachuk et al., Clinical applications of fluorescence in situ

hybridization", Genetic analysis techniques and applications vol. 8, 67-74, 1991). However, in practice 2 to 4% of normal interphase cells tested by FISH will show false-positive results due to the fact that the two probes colocalize by chance. An additional disadvantage of the current FISH protocols is that it is in practice necessary to know both chromosomes that are involved in the translocation as well as the relevant breakpoint regions of both chromosomes to define the nucleic acid probes enabling the detection of the specified translocation, while as yet unknown or ill-defined translocations originating from a well-known gene and an unknown partner gene remain undetected.

The present invention provides nucleic acid probes that can be used in diagnostic testing for chromosome aberrations which combine a high sensitivity and a high specificity. The probes provided by the invention can hybridise, *in situ* or *in vivo* or *in vitro* with complementary nucleic acid molecules such as (m)RNA or DNA, as e.g. transcribed by or found in (non-aberrant and/or rearranged) chromosomes. The present invention provides for each translocation analysis a distinct and balanced pair of nucleic acid probes. The probes are distinct in that they each hybridise to a different sequence specifically selected and flanking a distinct potential breakpoint in a non-aberrant chromosome. Furthermore, the pair formed by e.g. probe A and probe B is distinct from the pair formed by e.g. probe A and probe X. Furthermore, in above example probes A, B and X constitute three pairs, A-B, B-X and A-X. The probes in said pair are comparable or balanced in that they are designed to be of for example comparable size or genomic length with the final aim of facilitating the generation of signals of comparable intensity. In addition, said probes can be comparably labelled with reporter molecules resulting in signals of comparable intensity. In addition, said probes can

each be labelled with a different fluorochrome, facilitating detection on one spot of different colour when they colocalize when no aberration is detected. Also, said probes can be selected to react with a chromosome, at respective
5 complementary hybridisation sites that are located at comparable distances at each side of a breakpoint or breakpoint cluster region of a chromosome. The distinct and balanced pair of nucleic acid probes provided by the invention entails probes that are for example of comparable
10 size or genomic length, each probe of the pair for example being from 1 to 10 kb, or 7 to 15 kb, or 10 to 20 kb, or 15 to 30 kb, or 20 to 40 kb, or 30 to 50 kb, or 40 to 60 kb, or 50 to 70 kb, or 60 to 80 kb, or 70 to 90 kb, or 80 to 100 kb in length. By using such a distinct and balanced pair of
15 probes flanking a breakpoint region and not overlapping the corresponding fusion region, false-positive diagnosis in hybridisation studies is avoided. The invention further provides a distinct and balanced pair of nucleic acid probes, each being labelled with at least one different reporter
20 molecule. Nucleic acid probes can be labelled with chromophores or fluorochromes (e.g. FITC or TRITC) or by introducing a hapten such as biotin and digoxigenin. Fluorochrome labelled probes can be detected directly. Hybridisation with haptenised nucleic acid probes is followed
25 by indirect detection using chromophores, fluorochromes or enzymes such as peroxidase. The invention further provides a distinct and balanced pair of nucleic acid probes which are characterised in that both probes hybridise to a single corresponding nucleic acid molecule or its complementary
30 strand, or hybridise to one (non-aberrant) chromosome, or hybridise to a fragment thereof, possibly comprising the aberration, instead of two probes that hybridise separately to the two chromosomes that are involved in a given translocation, as currently used in hematology and oncology

in general (see for example Tkachuk et al., Science 250, 559-562, 1990; Tkachuk et al., Clinical applications of fluorescence in situ hybridization", Genetic analysis techniques and applications vol. 8, 67-74, 1991).

5 The invention further provides a distinct and balanced pair of nucleic acid probes which hybridise to said nucleic acid molecule at a genomic distance of no more than 100 kb, but preferably no more than 50 kb. In addition the invention provides a distinct and balanced pair of nucleic
10 acid probes which hybridise *in situ*, and can i.e. be used in diagnostic tests entailing FISH techniques. Furthermore, the invention provides a distinct and balanced pair of nucleic acid probes which probes each hybridise *in situ* under varying but generally low-stringent conditions to only a few DNA
15 molecules per cell. The nucleic acid probes composed of several DNA fragments are tested either on metaphase spreads or with Southern blotting for hybridisation sensitivity and specificity to select the probe on containing as little major repetitive sequences as possible, to avoid high background
20 staining. The nucleic acid probes are tested in fiber FISH (i.e. hybridisation on extended single DNA fibers immobilised on glass slides), prior to being employed in diagnostic testing, for mapping and checking their relative positions.

 The probes are tested for example to avoid using
25 probes hybridising two repetitive sequences. Probes can consist of sets of various oligonucleotides, thereby avoiding repetitive sequences present in a flanking region. Such sets are distinctly labelled, with separate or distinct reporter molecules for each probe (or set of oligonucleotides) that is
30 aimed at the respective flanking region. Such probes can each consist of multiple labelled oligonucleotides, each hybridising to a distinct area in a flanking region. One probe can for example contain from 10 up to 200 of such oligonucleotides, preferably from 50-150, each

oligonucleotide for example being 10-20 nucleotides long. For example, the intron-exon structure of the *MLL* gene is described in the Br J Haematol 1996;93:966-972. The manuscript also shows that most breakpoints in the *MLL* gene are located between exon 9 and exon 14. PNA containing probes can be designed in exon 3 to 8 for the "upstream FISH probe" and in exon 15 to 31 for the "downstream FISH probe". Particularly exon 4 and exon 28 are important for probe design, because these two exons are rather large and therefore can contain most of the PNA probes. PNA oligonucleotides can be synthesized for example for their capacity to hybridise with said exon 4 or exon 28 from the 119Q3 target gene and used in one cocktail as probe for one flanking region.

The invention further provides the use of said distinct and balanced pair of probes in diagnostic testing for chromosome aberrations. The pair of probes according to the invention can be used in the detection of nucleic acid comprising the aberration or fragments of the aberration, or in the detection of cells, *in situ* or *in vitro*, comprising the chromosome aberration. The invention thus provides a pair or pairs of distinct and balanced probes which can be used in the detection of disorders or diseases related to chromosomal aberrations, i.e. malignancies, such as haematopoietic malignancies as further explained below. Furthermore, the invention provides a diagnostic kit or assay comprising a pair of nucleic acid probes according to the invention which can be used in the detection of disorders or diseases related to chromosomal aberrations, i.e. malignancies, such as haematopoietic malignancies with such a diagnostic kit or assay provided by the invention it is e.g. possible to monitor the effects of therapy and detect minimal residual disease or detect early relapse of cancer. One can also identify the origin of bone marrow cells following bone

marrow transplantation. One can also detect viral sequences, and their localisation in the chromosome, in cells. More in detail the present invention is described while referring to molecular detection of chromosome aberrations in

5 hematopoietic malignancies but is widely applicable for analysis of chromosome aberrations in general.

The development of reliable probes for detection of well-defined or even ill-defined chromosome aberrations in hematological malignancies is described as non-limiting

10 example to illustrate the invention. Such probes can be used for diagnosis and for molecular classification of the involved malignancies. The new probes can be used in diagnostic testing in several types of hematological malignancies with increased sensitivity, specificity, and

15 efficacy of analysis.

Each year world-wide many cases of hematopoietic malignancies are being diagnosed. In the European Union (~375 million inhabitants) this concerns ~98,000 patients per year. The estimated number of patients in the USA (~250 million

20 inhabitants) is ~65,500 per year. The majority of hematological malignancies are of lymphoid origin: acute lymphoblastic leukemias (ALL), chronic lymphocytic leukemias, most malignant lymphomas, and multiple myelomas. The non-Hodgkin's lymphomas (NHL) form the largest group,

25 representing approximately half of all hematopoietic malignancies. Furthermore, European epidemiological studies show that the incidence of NHL is gradually increasing (~5% per year), which indicates that NHL poses a significant public health problem in Europe and most probably throughout

30 the Western world. Although the annual number of patients diagnosed with ALL is smaller than for NHL, ALL has a high prevalence in children, representing the most frequent malignancy in childhood.

Lymphoid malignancies consist of a broad range of ~25 different disease entities, which differ in clinical presentation, prognosis, and treatment protocols. These disease entities have been defined in the recent Revised European American Lymphoid neoplasm (REAL) classification. In this classification the lymphoid malignancies are divided in B-cell malignancies (~90%) and T-cell malignancies (~10%).

The diagnosis and classification of lymphoid malignancies is generally based on cytomorphology and histomorphology, complemented with immunophenotypic information via flow cytometry and/or immunohistochemistry. This immunophenotypic information appears to be valuable for classification of lymphoid malignancies, such as the classification of ALL into pro-B-ALL, common-ALL, pre-B-ALL, and several types of T-ALL. In mature B-cell malignancies with immunoglobulin (Ig) expression the diagnosis can be supported by immunophenotypic clonality assessment via detection of single Ig light chain expression, i.e. the distribution of Igk and Igλ positive B-cells, which is heavily skewed in case of a B-cell malignancy.

The value of clonality assessment is based on the fact that all cells of a malignancy have a common clonal origin. In lymphoid malignancies this is reflected by the presence of identically (clonally) rearranged Ig and T cell receptor (TCR) genes: clonal Ig and/or TCR gene rearrangements are found in most (90-95%) immature lymphoid malignancies and virtually all (>98%) mature lymphoid malignancies. Therefore molecular clonality analysis of Ig and TCR genes is highly suitable for discrimination between monoclonal (malignant) and polyclonal (reactive) lymphoproliferations. Suspect lymphoproliferations should therefore be subjected to molecular clonality assessment.

During the last decade the knowledge about genetic aberrations in hematopoietic malignancies has considerably

increased, especially in acute leukemias and NHL. Currently, well-established chromosome aberrations are found in 35-40% of ALL and in 30-40% of NHL. These chromosome aberrations can be used as alternative or additional markers for molecular
5 clonality assessment. More importantly, these chromosome aberrations appear to be relevant classification markers, which supplement the currently used morphological and immunophenotypic classification systems. It has been clearly demonstrated that several genetic aberrations are associated
10 with a favourable prognosis, whereas others are associated with poor prognosis, such as t(4;11) in pro-B-ALL and t(9;22) in common-ALL. Several treatment protocols have started to use this information for stratification of treatment. Therefore it can be anticipated that rapid and reliable
15 detection of well-defined genetic aberrations will become essential in the diagnosis and management of hematopoietic malignancies.

Several different types of chromosome aberrations have been identified in ALL and NHL. The chromosome
20 aberrations in precursor-B-ALL mainly concern translocations, which result in fusion genes, encoding for fusion proteins with new or modified functions. Examples include the E2A-PBX and BCR-ABL fusion proteins, resulting from t(1;19) and t(9;22), respectively. Another important chromosome region,
25 the 11q23 region with the *MLL* gene, is involved in several types of translocations in acute leukemias. In these 11q23 translocations different partner genes are involved, leading to different fusion proteins. One of them is t(4;11), which is observed in ~70% of infant acute leukemias. Many
30 chromosome aberrations in T-ALL and NHL involve Ig or TCR gene sequences in combination with oncogene sequences. These chromosome aberrations do not give rise to fusion proteins, but result in increased or stabilized expression of the involved oncogene, thereby contributing to uncontrolled

growth. They occur at relatively high frequency in particular disease categories, such as t(14;18) with involvement of the *BCL2* gene in ~90% of follicular lymphomas and t(11;14) with involvement of the *BCL1*/Cyclin D1 gene in ~70% of mantle cell lymphomas.

From origin cytogenetic analysis of chromosomes has been the standard technique for detection of chromosome aberrations. This technique needs the presence of cells in metaphase, which generally requires various cell culture systems, dependent on the type of malignancy. The success rate for obtaining reliable karyograms is highly dependent on the type of malignancy and the experience of the laboratory and ranges from less than 50% to over 90%. Furthermore, some chromosome aberrations can not or hardly be detected by cytogenetic analysis such as *TAL1* deletions in T-ALL and t(12;21) in precursor-B-ALL. Therefore in case of well-established chromosome aberrations the labor-intensive and time-consuming classical cytogenetics is now being replaced by molecular techniques. As said, molecular analysis of genetic aberrations can be performed with Southern blotting, polymerase chain reaction (PCR) techniques, and FISH techniques.

Southern blot analysis has long been the most reliable molecular method for detection of well-established chromosome aberrations, but this technique is dependent on the availability of suitable DNA probes, which recognize all relevant breakpoint cluster regions of the involved chromosome aberrations. The latter probably explains why *BCL2* and *BCL1*/CyclinD1 gene aberrations are detectable by Southern blotting in only 75% of follicular lymphomas and in only 50% of mantle cell lymphomas, respectively. Furthermore, Southern blot analysis is time-consuming and requires relatively large amounts of high-quality DNA derived from fresh or frozen cell samples.

Over the last five years, PCR-based techniques have been developed as alternatives for Southern blotting. PCR techniques have the advantage that they are rapid and require minimal amounts of medium-quality DNA, which might even be obtained from formalin-fixed paraffin-embedded tissue samples. Also mRNA can be used after reverse transcription (RT) into cDNA. RT-PCR is especially valuable in case of chromosome aberrations with fusion genes and fusion transcripts, such as frequently seen in precursor-B-ALL and in t(2;5) in anaplastic large cell lymphoma. Despite these obvious advantages, the broad application of PCR techniques for detection of chromosome aberrations in hematopoietic malignancies is hampered by several problems. False-negative PCR results can be obtained if the DNA or mRNA from formalin-fixed paraffin-embedded tissue samples is less optimal than anticipated, or when primers are mismatching. False-positive results might be obtained due to cross-contamination of PCR products between samples from different patients; especially in case of RT-PCR studies of fusion gene transcripts it might be difficult to exclude false-positive results. Finally, routine PCR analysis can only be used to study relatively small fusion regions of chromosome breakpoints (<2 kb). This implies that multiple oligonucleotide primer sets are needed to cover the most important breakpoint and fusion regions, whereas it will be difficult to study large breakpoint or fusion regions (>10 kb). This explains the lower detectability of chromosome aberrations, and thus again the presence of false-negative results, at the DNA level by PCR as compared to Southern blotting.

A major advantage of FISH techniques as compared to cytogenetic analysis, Southern blotting, and PCR analysis is that FISH can be performed on interphase nuclei of all kind of tissue and cell samples and that there is no need for extraction of DNA or mRNA. In FISH techniques generally large

DNA probes (>25 kb) are used, which are located around the breakpoint regions of the two chromosomes of the studied chromosome aberration. This implies that FISH probes can scan much larger regions than Southern blot probes or PCR primers.

5 This advantage is especially important for detection of breakpoints outside the traditional breakpoint cluster regions. Furthermore the use of large fluorescently-labeled DNA probes allow direct and rapid visualization of deletions and translocations of the studied gene regions. Application
10 of the latest generation of fluorescent microscopes with multiple fluorochrome filter combinations, CCD camera, and appropriate computer software allow the combined use of multiple FISH probes, which are labeled with different fluorochromes.

15 The availability of suitable probes is the main limiting factor in using FISH technology for detection of chromosome aberrations. Thus far, generally cosmid clones, YAC clones, or other cloned DNA fragments have been used without specific selection or modification of these probes.
20 For many of these probes the position in the genome is not precisely known; they often even overlap with breakpoint cluster regions, and they often contain repetitive sequences which cause high background staining. Furthermore, translocations are generally detected by use of two different
25 probes, one for each of the involved chromosomes; these two probes are assumed to colocalize in case of a translocation, but show separate signals if no translocation is present. However, in practice 2 to 4% of normal interphase cells will show false-positive results due to the fact that the two
30 signals colocalize by chance.

For routine applicability of FISH techniques or other probe analysis assays or kits for the detection of chromosome aberrations in the diagnosis and classification of

hematopoietic malignancies, it is necessary to design distinct and balanced probes.

1. The probes of the invention are selected to form a distinct and balanced pair of nucleic acid probes; size of the probes is each within certain limits of the genomes to be detected (e.g. 1-10, or 10-30, or 20-40, or 30-50, or 40-60 kb), with the final aim that the intensity of the fluorescent signals of the various probes is comparable.

2. In an additional embodiment of the invention the position of the probes constituting the pair is determined precisely, i.e. no overlap with breakpoint cluster regions, the relevant breakpoints are preferably located within 50 kb or preferably even within 25 kb of either probe, and an additional probe pair has to be designed, if two breakpoint regions of a particular chromosome aberration are separated for more than 30-50 kb depending on the exact position of the probes.

3. In a further embodiment the nucleic acid probes do not contain (major) repetitive sequences, and do not cross-hybridise, which results in high background staining. For this reason the nucleic acid probes composed of several fragments can be tested either on metaphase spreads or with Southern blotting for hybridisation sensitivity and specificity.

4. The nucleic acid probes can alternatively, or additionally be tested in fiber FISH prior to being employed in diagnostic testing, for mapping and checking their relative positions.

5. It has additionally been found that detection of chromosome breakpoints becomes easier and more reliable, if two separate probes, labelled with two different fluorochromes, constituting said pair are designed around one of the breakpoint regions of a chromosome aberration. This will lead to colocalization of the signals if no breakpoint is present. However if a breakpoint occurs in the studied breakpoint region, the two differently labeled probes will result in two separate signals.

6. In addition, the design of a third probe (labeled with a third fluorochrome) and thus the design of two additional distinct pairs of probes for the partner gene of the chromosome aberration allows precise identification of the chromosome aberration.

Chromosome aberrations found in malignancies are useful for molecular classification, such as in case of acute leukemias, malignant lymphomas and solid tumors (Table 1). However, several of these aberrations are more important than others, because of their high frequency or because of their prognostic value. For instance, t(14;18) occurs frequently in NHL, whereas t(12;21) is frequently found in childhood precursor-B-ALL. On the other hand, translocations involving the *MLL* gene in the 11q23 region represent a poor prognostic factor and the presence of 11q23 (*MLL* gene) aberrations is already in use as an important factor for stratification of treatment in acute leukemias. Also t(9;22) in ALL has a poor prognosis and is used for treatment stratification

The *MLL* (for myeloid-lymphoid leukemia or mixed-lineage leukemia) gene in chromosome region 11q23 is involved in several translocations in both ALL and acute myeloid leukemias (AML). In these translocations the *MLL* gene, encoding a protein that shows homology to the *Drosophila* trithorax gene product, is fused to partner genes on

different chromosomes. To date at least ten partner genes have been identified. Some of these translocations, like the $t(4;11)(q21;q23)$, $t(11;19)(q23;p13)$ and $t(1;11)(p32;q23)$, predominantly occur in ALL, whereas others, like

5 $t(1;11)(q21;q23)$, $t(2;11)(p21;q23)$, $t(6;11)(q27;q23)$ and $t(9;11)(p22;q23)$ are more often observed in AML. Other types have been reported in ALL as well as AML. Treatment-induced AML with 11q23 aberrations can arise in patients previously treated with topoisomerase II inhibitors. Rearrangements

10 involving the 11q23 region occur very frequently in infant acute leukemias (around 60-70%), and to a much lesser extent in childhood and adult leukemias (each around 5%). *MLL* gene rearrangements, especially the $t(4;11)$, have been shown to be a poor prognostic factor in infant leukemias, resulting in a

15 3-year overall survival of 5% as compared to 85-90% in cases with germline *MLL* genes.

The large *MLL* gene (>100 kb) consists of 21 exons, encoding over 3900 amino acids. Breakpoints in the *MLL* gene are clustered in a 8.5-9 kb region that encompasses exons 5-

20 11. Because of its relatively small size, this breakpoint region is easily accessible for molecular detection of translocations. By choosing two distinctly-labeled FISH probes in the sequences flanking the breakpoint region, any translocation involving the 11q23 region can be detected on

25 the basis of segregation of the two fluorochrome signals, whereas the two fluorochromes colocalize when no rearrangement in the *MLL* gene has occurred. Furthermore, the use of a third fluorochrome for probes directed against partner genes enables the identification of the precise type

30 of translocation. This two-step approach of FISH analysis guarantees efficient and direct detection of all aberrations involving the 11q23 (*MLL* gene) region in the first step, whereas in the second step the type of 11q23 translocation can be determined.

Chromosome aberrations in lymphoid malignancies often involve Ig or TCR genes. Examples include the three types of translocations (t(8;14), t(2;8), and t(8;22)) that are found in Burkitt's lymphomas, in which the *MYC* gene is coupled to
5 Ig heavy chain (*IGH*), Ig kappa (*IGK*), or Ig lambda (*IGL*) gene segments, respectively. Another common type of translocation in this category is the t(14;18)(q32;q21) that is observed in ~90% of follicular lymphomas, one of the major NHL types. In this translocation the *BCL2* gene is rearranged to regions
10 within the *IGH* locus within or adjacent to the JH gene segments. The result of this chromosome aberration is the overexpression of the *BCL2* protein, which plays a role as survival factor in growth control by inhibiting programmed cell death.

15 The *BCL2* gene consists of only three exons, but these are scattered over a large area. Of these the last exon encodes a large 3' untranslated region (3' UTR). This 3' UTR is one of the two regions in which many of the t(14;18) breakpoints are clustered and is called "major breakpoint
20 region" (mbr); the other breakpoint region involved in t(14;18) translocations, is located 20-30 kb downstream of the *BCL2* locus and is called the "minor cluster region" (mcr). A third *BCL2* breakpoint area, the vcr (variant cluster
25 region), is located at the 5' side of the *BCL2* locus and is amongst others involved in variant translocations, i.e. t(2;18) and t(18;22), in which *IGK* and *IGL* gene segments are the partner genes.

By choosing a set of FISH probes that are located in the regions upstream of the mbr region and downstream of the
30 mcr region, translocations in these regions can be detected upon segregation of the fluorochrome signals. An additional set of FISH probes is designed for the vcr region, since the distance between the vcr region and the other two breakpoint clusters is far too large (~400 kb) to use the same probes.

As a second step in all these approaches, FISH probes in the *IGH*, *IGK*, and *IGL* genes are used for identification of the exact type of translocation.

Several types of nucleic acid probes can be employed in FISH technology as provided by the invention for detection of chromosome aberrations. Each of these probe types has its own characteristic features and advantages, together constituting a complementary approach, i.e. cosmid, PAC, or YAC derived probes, PCR-based probes, or PNA-based probes.

Clones obtained from cosmid, PAC or YAC libraries constitute large probes that, when labeled with fluorochromes, result in appropriate signals upon hybridization (Gingrich et al., 1996). However, conventionally as the precise position of these probes is unknown, there often is a risk of overlap with the breakpoint cluster region of the involved chromosome aberration, if no further selection or modification of these probes is performed. Furthermore, such large probes often contain repetitive sequences, which cause high background staining. Distinct and balanced pairs of probes comprising cosmid, PAC or YAC probes that are designed to react with the flanking regions upstream and downstream of the breakpoint area on one of the involved chromosomes can therefore be exactly positioned in fiber FISH experiments or by use of Southern blotting using small well-defined inclusion and exclusion probes which are designed around the breakpoint area avoiding said overlap with the breakpoint cluster. The presence of potential repetitive sequences is excluded via Southern blot analysis of genomic DNA.

Probes that are generated by PCR have the additional advantage that they can be positioned exactly, however for this approach sequence information is required at least in the areas for designing the target-specific PCR primers for producing the probes. Once generated the PCR products are

checked for the presence of repetitive or cross-hybridizing sequences that hamper specific detection of the flanking regions upstream and downstream of the involved break point cluster. PNA-based probes for FISH technology comprise multiple (e.g. 50-150) distinct PNA oligonucleotides each of which shows a typical size of 5-40, more typically 10-25 nucleotides and which together produce an appropriate signal for detection of chromosome aberrations using FISH technology. PNA probes, having a neutral peptide backbone to which the four deoxynucleotides are coupled are stable nucleic acid fragments that hybridize to complementary nucleic acid sequences with high affinity (Egholm et al., 1993; Corey, 1997). Due to the fact that mismatches strongly influence PNA hybridization, sequence specificity of PNA recognition can be easily achieved, thereby rendering PNA probes highly selective probes to be used in FISH technology. PNA probes have now been used in a variety of applications including *in situ* hybridization to highly repetitive centromeric or telomeric sequences (Corey, 1997). Thusfar, only a single PNA oligonucleotide directed against repeated sequences was sufficient for appropriate signal intensities. The design of a balance pair of nucleic acid probes comprising multiple (e.g. 50-150) distinct PNA oligonucleotides directed against target sequences in the flanking regions of a breakpoint cluster provides detection of chromosome aberrations as well as with other nucleic acid probes.

Applicability of the various types of probes

Each of the before mentioned types of FISH probes has its specific applicability, but together they constitute complementary and partly overlapping strategies.

The *MLL* gene in chromosome region 11q23 is an example of a detectable region that is involved in several translocations in both ALL and AML (Table 1), providing a perfect example of a chromosome aberration for which PCR-based or PNA-based FISH probes can preferably be designed. Because the breakpoint area in the *MLL* gene is so tightly clustered with ample exons available in the flanking regions upstream and downstream of the breakpoint cluster, sequence-based design and production of distinct and balanced pairs of PCR-based and/or PNA-based FISH probes is very useful in this chromosome region. Design of precisely positioned cosmid or PAC clones could be useful as an alternative or additional strategy.

The *BCL2* gene area is an example that is involved in several chromosome aberrations in malignant lymphomas (Table 1) which contains several breakpoint areas which are located outside the coding sequence at the 5' and 3' sides of the *BCL2* locus and which are lying far apart. The *BCL2* locus therefore exemplifies a gene involved in chromosome aberrations for which distinct and balanced pairs of FISH probes are more difficult to generate via PCR and/or via pooling of PNA oligonucleotides, as less sequence information is available. In such chromosome aberrations distinct and balanced pairs of cosmid, PAC, and/or YAC derived FISH probes can be employed after careful selection and modification of the exact position.

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Table 1. Examples of chromosome aberrations in malignancies, that are detectable with a distinct and balanced pair of nucleic acid probes of the invention.

5	Translocation	Involved genes	Primary target gene for FISH probe design	Occurrence per disease category
10	<i>Acute leukemias</i>			
	t(4;11) (q21;Q23)	<i>MLL-AF4</i>	<i>MLL</i> gene	- 70% of infant ALL
	t(11;19) (q23;p13)	<i>MLL-ENL</i>		- 5-7% of ALL
	t(6;11) (q27;q23)	<i>MLL-AF6</i>		- 5-6% of AML
	t(9;11) (p22;q23)	<i>MLL-AF9</i>		
15	<i>Malignant lymphomas</i>			
	t(14;18) (q23;q21)	<i>BCL2-IGH</i>	<i>BCL2</i> gene	- 90% of follicular NHL
	t(2;18) (p12;q21)	<i>IGK-BCL2</i>		- 25% of immunoblastic NHL
	t(18;22) (q21;q11)	<i>IGL-BCL2</i>		- 25% of diffuse large cell centroblastic NHL
20				
	<i>Solid tumors</i>			
25	t(11;22) (q24;q12)	<i>EWS-FLI1</i>	<i>EWS</i> gene	- 5-10% of B-CLL
	t(21;22) (q22;q12)	<i>EWS-ERG</i>		>95% of Ewing sarcoma
	t(7;22) (p22;q12)	<i>EWS-ETV1</i>		

Claims

1. A pair of nucleic acid probes of comparable size, each preferably being from 1 to 100 kb, more preferably each being from 1 to 10 kb, or 7 to 15 kb, or 10 to 20 kb, or 10 to 30 kb, or 20 to 40 kb, or 30 to 50 kb, or 40 to 60 kb, or 50 to 5 70 kb, or 60 to 80 kb, or 70 to 90 kb, or 80 to 100 kb, and flanking a potential breakpoint in a chromosome.
2. A pair of nucleic acid probes according to claim 1, each being labeled directly or indirectly with at least one reporter molecule.
- 10 3. A pair of nucleic acid probes according to claim 2 wherein the reporter molecule is selected from the group consisting of enzymes, chromophores, fluorochromes, haptens (such as biotin or digoxigenin).
4. A pair of nucleic acid probes according to any of 15 claims 1 to 3 characterised in that probes hybridise to a single corresponding nucleic acid molecule.
5. A pair of nucleic acid probes according to claim 4 wherein the corresponding nucleic acid molecule is at least a fragment of a chromosome.
- 20 6. A pair of nucleic acid probes according to claim 5 wherein the chromosome is not aberrant.
7. A pair of nucleic acid probes according to any of claims 4 to 6 which hybridise to said nucleic acid molecule at a genomic distance of no more than 100 kb, but preferably 25 no more than 50 kb.
8. A pair of nucleic acid probes according to any of claims 1 to 7 which hybridise *in situ*.
9. A pair of nucleic acid probes according to any of the claims above which probes each hybridise *in situ* under low- 30 stringent conditions to only a few linear DNA molecules per cell.

10. Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of a nucleic acid molecule comprising a chromosome aberration.
11. Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of cells comprising a chromosome aberration.
12. Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of a disorder or disease caused by a chromosome aberration.
- 10 13. Use of a pair of nucleic acid probes according to any of claims 10 to 12 wherein the chromosome aberration is related to a malignancy.
14. Use of a pair of nucleic acid probes according to any of claims 10 to 12 wherein the chromosome aberration is related to a hematopoietic malignancy.
- 15 15. A diagnostic kit comprising at least a pair of nucleic acid probes according to any of claims 1 to 9.

INTERNATIONAL SEARCH REPORT

International Application No

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TKACHUK ET AL.: "Detection of bcr-abl fusion in chronic myelogeneous leukemia by in situ hybridization" SCIENCE, vol. 250, 1990, pages 559-562, XP002044140 see the whole document	1-15
X	TKACHUK D C ET AL: "CLINICAL APPLICATIONS OF FLUORESCENCE IN SITU HYBRIDIZATION" GENETIC ANALYSIS TECHNIQUES AND APPLICATIONS, vol. 8, no. 2, 1 April 1991, pages 67-74, XP000262526 see the whole document	1-15
X	EP 0 430 402 A (UNIV CALIFORNIA) 5 June 1991 see the whole document	1-15



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 96 17958 A (UNIV CALIFORNIA ;MEDICAL RES COUNCIL (GB)) 13 June 1996 see the whole document ---	1-15
A	THOMPSON C T ET AL: "CYTOGENETIC PROFILING USING FLUORESCENCE IN SITU HYBRIDIZATION (FISH) AND COMPARATIVE GENOMIC HYBRIDIZATION (CGH)" JOURNAL OF CELLULAR BIOCHEMISTRY. SUPPLEMENT, no. SUPPL. 17G, 1993, pages 139-143, XP000612761 see the whole document ---	1-15
A	US 5 538 869 A (SICILIANO MICHAEL J ET AL) 23 July 1996 see the whole document ---	1-15
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